ENHANCED RECOVERY, VIABILITY, AND PROLIFERATION OF HUMAN BLADDER EPITHELIAL CELLS AFTER CRYOPRESERVATION *IN VITRO*

by

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Abstract

Reconstruction of the bladder urothelium requires human bladder epithelial cells (hBECs), which are known to have an inconsistent recovery and growth rate following cryopreservation. This study aims to determine methods to increase recovery and rates of proliferation after cryopreservation. Specifically, our study looks to improve upon the current existing protocol of cryopreserving hBECs which allow for limited recovery and slow growth. Based on a review of prior literature, experiments were divided into two categories. One group of experiments focused primarily on the determining the optimal chemical composition of the freezing mixture by which hBECs were treated with prior to freezing, while maintaining physical parameters such as the cooling rate, cell density, and equilibration time. The other group of experiments focused on altering the above physical parameters that the cells were exposed to while maintaining a consistent freezing mixture consisting of DMSO and hBEC culturing medium. Analysis of the data demonstrated that cells treated with 10% DMSO and 10% PEG each had fairly high recoveries, and mixtures of the two resulted in consistent, and slightly higher recoveries. When ROCK inhibitor or PVA was added into the 10% DMSO freezing mixture, it was found that there were optimal concentrations of ROCK inhibitor and PVA resulted in significant increases in growth when added to the freezing mixture. In terms of physical parameters, increases in exposure to penetrating CPAs at room temperature resulted in decreases in recovery. Based on these results, alteration or addition of either of these factors allows for the increase in hBEC recovery following cryopreservation. Further research is needed to investigate the effects of cryopreservation on urothelial marker expression as well as clinical applications of cryopreserved hBECs in the reconstructive process of the urothelium.

Advisor: Dr. Anirudha Singh; **Reader:** Dr. David Gracias

Preface

This thesis will also be published in the future as a scientific paper.

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Acknowledgements

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Chapter 1: Introduction

Bladder cancer is currently the ninth most common cancer globally, and in the United States, it is now the fourth most common cancer in men[1]. Currently, bladder cancer is treated through a radical cystectomy, in which the entire bladder is removed. The most common treatment is the ileal conduit, in which a section of the small intestine takes the place of the bladder, as a reservoir for urine. While this procedure is currently the gold standard, there are many problems with incontinence as well as a reduced bladder volume[2–4]. However, recent advances in tissue engineering allow for the possible reconstruction of the bladder, one that may be functional and serve as a better alternative[5].

In order to artificially reconstruct the urothelial layer of a bladder, human bladder epithelial cells (hBECs) are ideally used in the place of urothelial cells. It has been previously reported that hBECs have a very slow proliferation time, a limited number of passages, and low viability after using conventional methods of cryopreservation. As a result, because of the inconsistency that occurs, there is typically an insufficient quantity of hBECs that allow for growth of the bladder; in addition, inconsistent growth and storage makes the possibility of a reconstructed functional bladder far less of a possibility. Because of this occurrence, we researched methods improve their viability after freezing, as well as to increase their rate of growth after being seeded in culture flaks with the use of cryoprotective agents and other substances reported in literature. Cryoprotective agents (CPAs) generally protect cells from injury during freezing, but have differ among themselves in their cryoprotective effects. In addition, there a number of factors outside of the immediate addition of CPAs that can influence the viability and proliferation of cells. To test these factors, we observe the effects of different CPAs on viability and proliferation, and then selected

the best penetrating CPA and non-penetrating CPA in the combinatory effects. After observing the best performing CPA mixture, we tested equilibration times, addition of ice recrystallization inhibitors (IRIs), storage times, cell density, and cooling rates. With these experiments, we were able to successfully determine the optimal conditions by which the hBECs were to be frozen. We believe that this method will be the foreground for which hBECs will be cryopreserved in the future, as well as to provide insight into the factors that have the most influence on cryopreservation of cells in general.

Chapter 2: Background

To understand the scope of our work, it is important to know the foundational concepts behind the methods. It is important to know the properties of hBECs and why there is a need for optimization, as well as understanding the mechanics of freezing using DMSO and other cryoprotective agents.

2.1 Human bladder epithelial cells and the bladder

The bladder is a unique organ in the urinary tract that serves as the reservoir for urine that is composed of three components major components: the detrusor muscle, the submucosa, and the urothelium[6]. In order to reconstruct a bladder, the submucosa and urothelium must be taken into account. The submucosa, which contains the stroma, can be developed using cells with similar properties that can be seeded on a scaffold. However, in order to construct the urothelium, human bladder epithelial cells are required on the luminal side of the scaffold[7]. hBECs are therefore are important in their use as models for the development of diagnostic methods, or for studying epithelial development and function[8,9]. However, hBECs grow very slowly, reaching 90% confluency in 10 days, compared to other mammalian cell lines that become confluent after a few

days. Despite the growing interest in using hBECs in research, cryopreservation and proliferation is consistently a problem. There are few reports on the cryopreservation of bladder epithelial cells, in which hBECs were frozen using a conventional freezing method, by using DMSO in cell medium. No further studies have been performed regarding the cryopreservation of bladder cells, as well as regarding the rate of proliferation and viability of the cells following cryopreservation.

2.2 Mechanisms of freezing

During the freezing process, the expansion of water into ice results in two types of injury, due to ice being a relatively pure substance, resulting in an increasingly concentrated solution from the reduced solvent. If cooling proceeds slowly, ice formation occurs extracellularly, causing the cell to lose water due to the osmotic gradient, and the excessive loss of water could result in osmotic injury. If cooling proceeds more rapidly, water has less time to leave the cell and freezing occurs intracellularly, resulting in mechanical stress on the cell membrane. Because of these two different types of injuries, there exists an optimal cooling rate at which both of these effects are minimized.

An alternative method of cryopreservation, known as vitrification, typically involves hydrated living cells to be cryopreserved in the absence of ice. Vitrification has recently become popular because it eliminates mechanical injury from ice, the need to find an optimal cooling rate, as well as the need to find the balance between intracellular ice formation and solute effects. However, vitrification almost always requires a high CPA concentration[10,11].

2.3 Cryoprotective Agents

Cryoprotective agents (CPAs) are divided into two classes: penetrating and non-penetrating. Penetrating CPAs (pCPAs) include dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, and propylene glycol, and are typically the commonly used type of CPA during

cryopreservation. They tend to be small, uncharged particles that penetrate the cell membrane in order to prevent intracellular ice crystal formation and dehydration by creating an environment that allows for the reduction of water within the cell. Because of their size, they are typically able to form hydrogen bonds with water to prevent ice crystallization and can control cell size changes.

Non-penetrating CPAs (npCPAs) tend to be larger molecules such as poly(ethylene glycol) or saccharides that do not pass through the cell membrane. They act by dehydrating the cell prior to freezing, reducing the amount of water that is lost in order to offset the osmotic imbalance caused by freezing. npCPAs also prevent damage to cells during recovery after cryopreservation by preventing a loss of necessary solutes from escaping the cell too rapidly. in addition, when paired with their penetrating counterparts, npCPAs can reduce the cell volume and increase intracellular osmolarity, further preventing intracellular ice formation[10]. To further investigate these cryoprotective effects on hBECs, we investigated a number of different CPAs based on prior research and applied Mazur's two-factor hypothesis, which posits that freezing is based on altered solution properties and intracellular freezing. Consideration of these two factors creates the potential for optimized conditions that minimizes both of these otherwise detrimental effects[12–14].

Dimethyl sulfoxide (DMSO), a penetrating CPA, has been the standard CPA for cell cryopreservation; however, it is highly toxic as it interferes with cell membranes and has potentially harmful effects in clinical applications[15]. Prior research has found that a combination of npCPAs can result in comparable, and sometimes, even higher recovery[16]. One study evaluated the cryopreservation of neonatal thymus tissue and stromal epithelial cells using various combinations of pCPAs and npCPAs together; it was found that a mixture of 95% dextran-40 and 5% DMSO provided the best cryopreservative capabilities in terms of viability and functionality[17].

2.4 Mazur 2-factor hypothesis

Chinese hamster tissue-cells were frozen at 0.4 M solutions of sucrose, DMSO, and glycerol. Cells that were frozen at a rate faster than the optimal freezing rate were more sensitive to slow arming, and it was found that the temperatures at which cells were killed were significantly different based on the rate at which they were frozen. Therefore, the Mazur 2-factor hypothesis postulates that freezing is based on two factors: altered solution properties in the extracellular and intracellular solution as well as the formation of intracellular ice. Consideration of these two factors during cryopreservation creates the potential for optimized conditions that minimizes the effects of both intracellular freezing and solute effects[12].

2.5 ROCK Inhibitor

Rho-associated coiled coil kinase (ROCK) proteins are typically associated with a range of cellular functions involving actin cytoskeleton reorganization, which includes cell adhesion, cytokinesis, and apoptosis. While the exact mechanism of ROCK inhibitor of Recently, ROCK inhibitor has been thoroughly investigated in the cryopreservation process of stem cells. Previous reports have shown that incorporation of 10 μM of ROCK inhibitor into the culture medium 24 hours after thawing resulted in higher proliferation as well as showing signs of differentiation[18,19]. More recently, another study researched the incorporation of ROCK inhibitor in both the culturing medium and the freezing medium; it was found that the incorporation of ROCK inhibitor freezing medium aided in the recovery as well[20].

2.6 Ice recrystallization inhibitors

A new type of cryoprotective agent, known as an ice recrystallization inhibitor (IRI), has been recently implemented in the cryopreservation process. IRIs are molecules that prevent or reduces ice nucleation and growth, specifically by adsorbing to the surface of ice crystals and

preventing them from growing despite the cooling temperature. Natural ice blockers include antifreeze proteins, or AFPs, which occur naturally in some Antarctic fish; however, ice blockers are typically thought of as synthetic chemicals, such as polyvinyl alcohol (PVA) [10,21–23]. It has been previously researched that low concentrations of PVA added to the freezing mixture prior to cryopreservation decreased the overall concentrations of glycerol or DMSO required; therefore, with the implementation of PVA as a part of the freezing process, there is potential for decreased mechanical injury from ice crystal formation as well as decreased toxic effects resulting from CPAs, leading to any possible increases in recovery.

Chapter 3: Experimental Methods

3.1 Materials

Primary Bladder Epithelial Cells (BdECs), Prostate Epithelial Cell basal media and Prostate Epithelial Cell growth kit were purchased from ATCC. BdECs obtained (Passage 2 - P2) were passaged (Passage 3 – P3) and stored in Liquid Nitrogen (LN_2) in Cryovials (10^6/vial) with 10% DMSO used as a cryoprotectant. The basal media and the growth kit were mixed together (referred to as complete media) and used as a growth medium for BdECs; the complete media was serum-free. The frozen cells were later grown in tissue culture flasks (P4) and harvested for further experiments. Dulbecco's Phosphate Buffer Saline (DPBS) was purchased form Millipore Sigma (USA). Trypsin (0.25%, 2.21 mM EDTA without Ca, Mg and Na bicarbonates with Phenol red), Plasma treated tissue culture flasks (75 cm^2) as well as 96 well plates were obtained from Corning. Trypan Blue solution (0.4%) was obtained from Gibco (USA). Live/Dead Cell viability assay kit (for mammalian cells) was obtained from Biovision (USA). DMSO (≥ 99.9%, anhydrous grade), Betaine (≥ 99.0%, molecular biology grade), L-Proline (≥ 99.5%, molecular biology grade), Trehalose (≥ 98.5%, cell culture grade), Dextran (average molecular weight 35,000-40,000) and

Poly (vinyl alcohol) (80% hydrolyzed, average molecular weight 9,000-10,000) were obtained from Sigma-Aldrich (USA). Poly (ethylene glycol)-400 (PEG-400) (reagent grade) was purchased from Merck (USA) and glycerol (≥ 99.0%, molecular biology grade) was purchased from Invitrogen (USA).

3.2 Cell culture

P3 cells were thawed and seeded onto 75 cm^2 tissue culture flasks (T75 flasks) at a density of 5000 cells/cm^2 as recommended by the ATCC with 10 mL of complete media. The flask was kept inside the incubator at 37°C, 95% air and 5% CO_2. The media was replaced with 10 mL fresh media 2 days after seeding following which it was renewed every alternate day. When the flasks were 80-90% confluent, they were trypsinized using Trypsin/EDTA followed by centrifugation to separate the supernatant and the remaining cell pellet was re-suspended in 1 mL of complete medium for further experiments.

3.3 Harvesting and freezing of cells

Confluent cells on tissue culture flasks were removed from the incubator and their surface was washed with spent media followed by treatment with 3 mL Trypsin and incubation for 5 min. The flask was tapped to detach the loosely attached cells. 5 mL complete media was added to the flask and the complete solution was aspirated out followed by washing with DPBS. The cell suspension was centrifuged and supernatant aspirated out to obtain only the cell pellet which was resuspended in 1 mL complete media. Cell in the suspension were counted using hemacytomter and Trypan Blue (0.4%, Gibco) (1:1 mixture of cell suspension and Trypan Blue) and the suspension was diluted to 1 mL per 2 million cells. CPAs were mixed with complete media to prepare the cryopreservation solution and 0.75 mL of the solution was added in 1 mL cryovials to which 0.25 mL of the cell suspension was added to make final volume to 1 mL, cell density to 0.5

million cells/cryovial and total CPA concentration to 10% (w/v or v/v). The cryovials were allowed to equilibrate for 15 minutes following which they were transferred freezing. Multiple methods were used for freezing the cells, the description of which is given in later sections.

Table 1.

Cryoprotective agent-medium mixture composition.

Cryoprotective agent	Composition (+ 90% hBEC culture medium/mL)
DMSO	10% v/v DMSO
Glycerol	10% v/v Glycerol
Betaine	10% w/v Betaine
L-Proline	10% w/v L-Proline
PEG	10% v/v PEG
Dextran	10% w/v Dextran
Trehalose	10% w/v Trehalose

3.4 Thawing and reseeding of cells

The cryovials were removed from the liquid N_2 tank and kept in a bead bath for 5 minutes (ice pellet completely disappeared by that time). The solution in each cryovial was transferred in 5 mL complete media and centrifuged at 1000 rpm for 5 min. After centrifugation, the supernatant was aspirated out and the pellet was resuspended in 5 mL complete media followed by centrifugation at 1000 rpm for 5 min. After aspirating out the supernatant and resuspending the pellet in 1 mL media, the cells were counted using hemocytometer. Appropriate number of cells were isolated from the suspension and seeded in 96 well plates with 200 μL of complete media in each well.

3.5 Cell counting for post thaw recovery test

20 µL of the cell suspension was mixed with 80 µL of hBEC media as well as 100 µL of Trypan blue and injected into the hemocytometer. The average of the four squares was multiplied by a factor of 10^5 to account for the dilution in order to obtain the final cell count. The counting process was repeated three times per vial frozen, to minimize error.

3.6 Viability of cells for observation of cell proliferation

Viable cells are counted using Live/Dead Assay kit and EVOS FLAuto 2 microscope under GFP (for live cells) and RFP (for dead cells) filter. The staining solution is prepared in accordance with the protocol mentioned on the website. One-hundred µL of the spent media is removed from all the wells to be analyzed followed by addition of 25 µL of the staining solution in each well. The wells are imaged under the microscope after gentle mixing followed by an incubation period of 15 min. The images obtained are processed with ImageJ to obtain the cell count. The counting method is validated by visually counting the cells in some wells and comparing with the results generated from ImageJ. A macro was then developed to automate the validated image processing method and used to analyze all the images.

3.7 Statistics

Three samples were used to obtain the mean and standard deviation for each data point. To compare the data between multiple groups, a two-way ANOVA test was used; otherwise, for comparisons against a positive control, a one-way ANOVA test was used. Data were considered statistically significant when $p < 0.05$.

Chapter 4: Results

Chemical composition of freezing solution

4.1 Screening of penetrating and non-penetrating CPAs for recovery and initial viability

Due to the limited understanding of the cryopreservation of hBECS, CPAs were selected based on prior cryopreservation research performed on different cell lines, as well as what has been previously seen in nature. To test the most appropriate CPA, hBECs were treated with a freezing solution of which the CPA concentration was consistently 10% (w/v or v/v), or with no CPA. hBECs were cryopreserved for seven days prior to thawing, and initial cell recovery was measured immediately after thawing using a hemocytometer and trypan blue staining. As shown in Figure 1, no significant difference in cell viability was observed between DMSO, PEG, and trehalose. However, the recovery of hBECs that were treated with glycerol, betaine, L-proline, and dextran were comparable to one another, and significantly lower than those treated with DMSO, PEG, and trehalose.

The number of viable cells was significantly lower in samples treated with glycerol, betaine, L-proline, and dextran compared to that of cells treated with DMSO, PEG, or trehalose. While the number of viable cells was slightly lower when treating cells with trehalose compared to DMSO and PEG it still performed significantly better than the majority of the CPAs. Based on the results of initial recovery, DMSO would be the standard pCPA that would be tested in further experiments; in addition, PEG and trehalose would be selected as the npCPAs that would be tested in CPA mixture experiments.

Table 2.

Post-thawing recovery cell count per milliliter.

CPA	Initial seeded cells	Recovered hBECs (cells/mL)	Recovered hBECs (%)
DMSO	500,000	366666 ± 28867	73.3 ± 5.8
Glycerol	500,000	150000 ± 40824	30.0 ± 8.2
Betaine	500,000	112500 ± 25000	22.5 ± 5.0
L-Proline	500,000	62500 ± 25000	12.5 ± 5.0
PEG	500,000	350000 ± 100000	70.0 ± 20.0
Dextran	500,000	112500 ± 25000	22.5 ± 5.0
Trehalose	500,000	275000 ± 95742	55.0 ± 19.1
No CPA	500,000	87500 ± 47871	17.5 ± 9.6

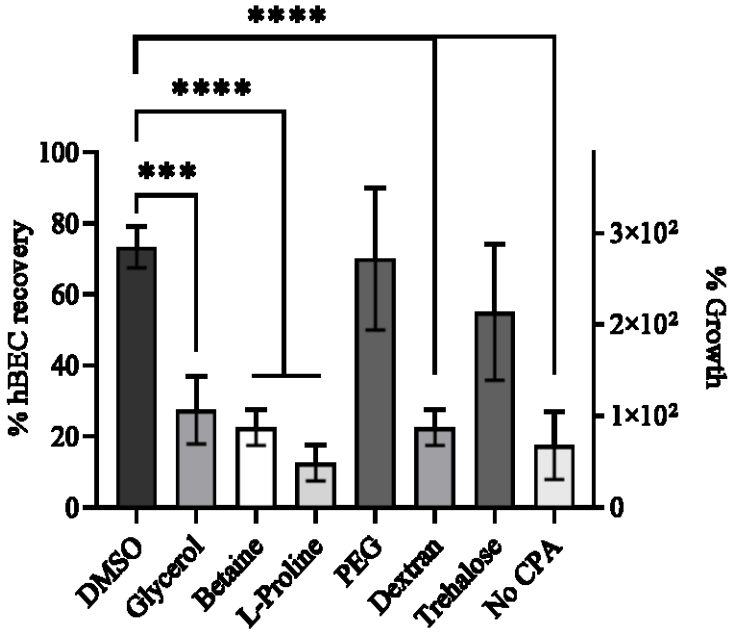

Figure 1. hBEC initial post thaw recovery after cryopreservation with different CPAs. Eight cryovials containing 0.5 x 10^6 hBECs were each frozen with a different CPA. The figure above is a graphical representation of the number of cells recovered immediately after thawing from cryopreservation for seven days at -140°C. ***p < 0.001 and ****p < 0.0001 compared to cells frozen with 10% DMSO.

4.2 Screening of penetrating and non-penetrating CPAs based on cell growth

Because trypan blue staining only provided a measure of initial viability, proliferation and continued viability was measured using live/dead staining and an EVOS FLAuto 2 microscope. hBEC viability was assessed by the amount of time that was required for hBECs to reach confluency. Two days after seeding in the 96 well plate, cells treated with DMSO, dextran, and no CPA displayed recoveries lower than cells treated with other types of CPAs. hBECs treated with glycerol, on the other hand, while initially showing limited recovery immediately after thawing, resulted in significant growth two days after seeding and frequently showed the significant growth throughout the eight-day growth period. DMSO, on the other hand, showed the greatest initial viability among the pCPAs, but resulted in significantly less proliferation compared to glycerol and L-proline. However, by the eighth day, DMSO and trehalose showed the greatest proliferation among the CPAs. It was also observed that hBECs treated with pCPAs showed the greatest percentage in growth between the fourth and fifth day after seeding, while hBECs treated with npCPAs showed the greatest growth between the sixth and eighth day after seeding.

From these data, we concluded that, based on proliferation data, the two best performing pCPAs were DMSO and glycerol. As shown in Figure 1 and 3, hBECs treated with DMSO resulted in both the highest recovery immediately after thawing and the greatest overall proliferation after the eight-day growth period. Cells treated with glycerol, on the other hand, frequently showed significant growth during the period, with an overall cell count similar to DMSO. Among the npCPAs, PEG and trehalose were the best performing for the hBECs; while cells treated PEG resulted in a higher immediate post-thawing recovery, cells treated with trehalose resulted in greater proliferation by the end of the eighth day.

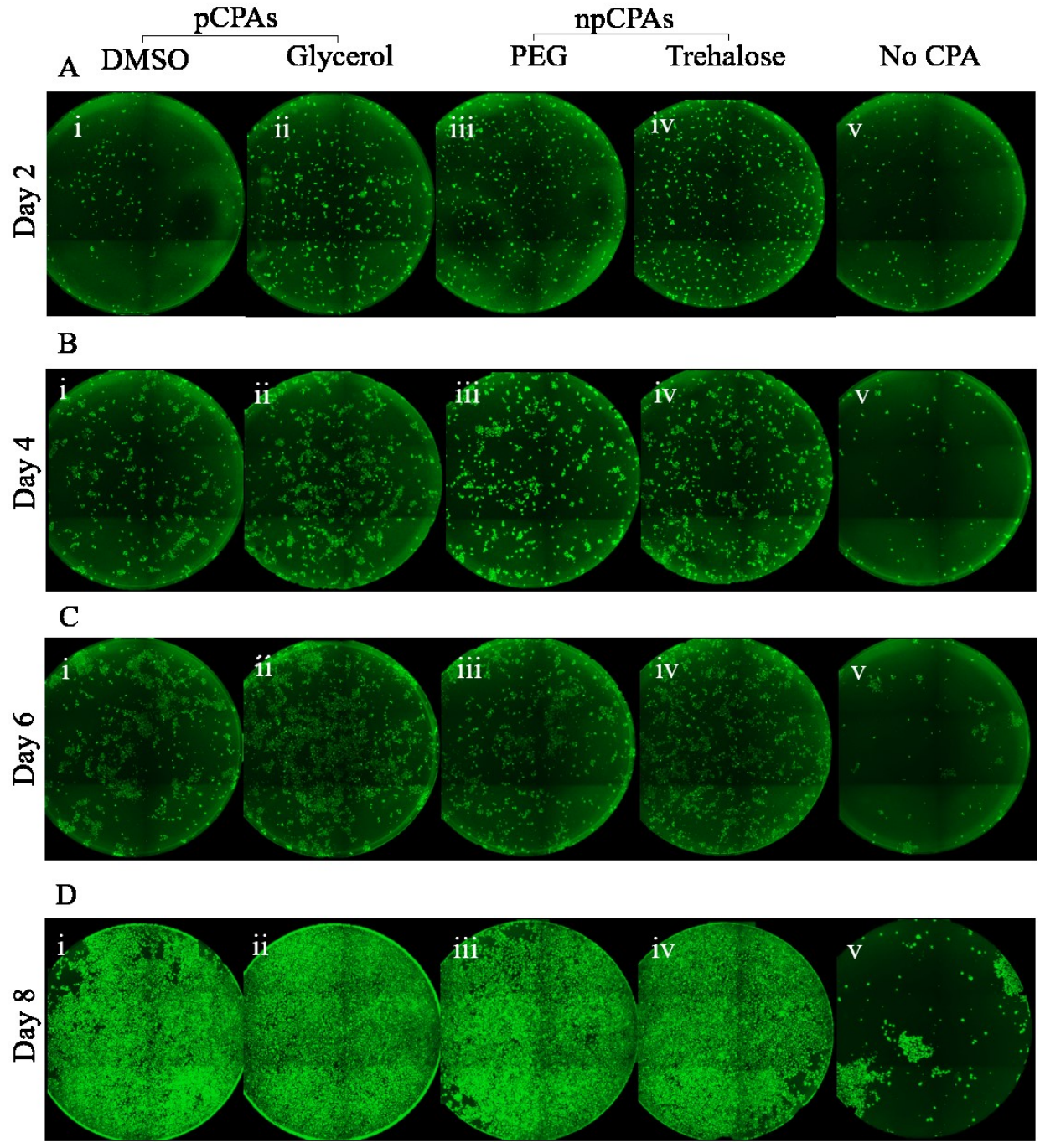

Figure 2. hBEC growth at two (A), four (B), six (C) and eight (D) days. Cells were treated with (i) DMSO, (ii) glycerol, (iii) PEG, (iv) trehalose, or (v) no CPA and seeded in a 96 well plate and imaged at 4x magnification with a fluorescent microscope. Prior to imaging, hBECs were stained with live/dead staining dye.

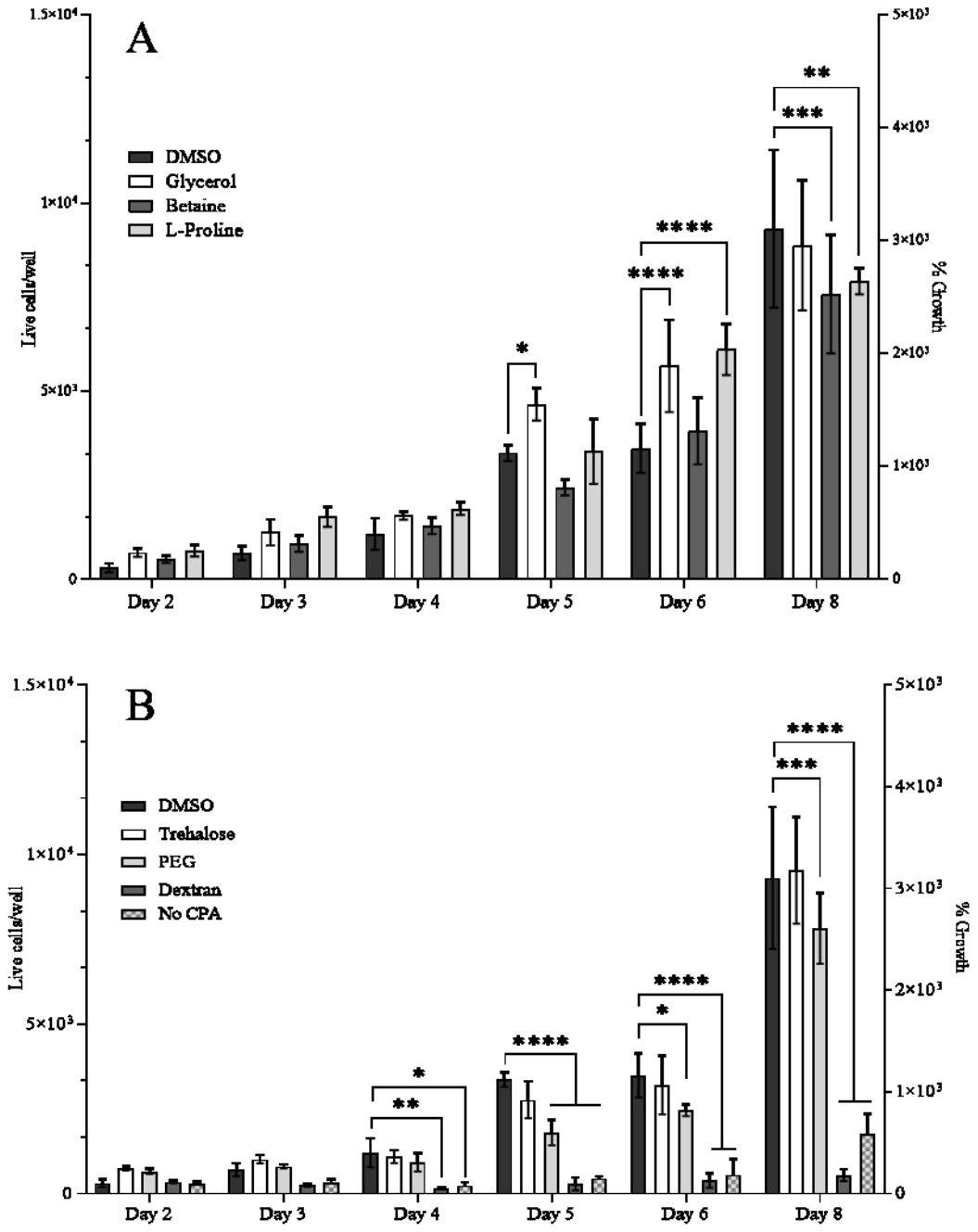

Figure 3. Growth of hBECs cryopreserved with various CPAs over an 8-day period. hBECs were resuspended and cryopreserved with either A) pCPAs or B) npCPAs Cells were seeded in 96 well plates at a density of 5000 cells/cm^2 and imaged two days after seeding to allow for adhesion and growth on the surface. hBECs were imaged at 4x magnification and counted using ImageJ.

4.3 Effect of CPA mixtures on recovery

Previous research has shown that cells treated with a freezing mixture of containing both penetrating and non-penetrating CPAs resulted in higher recoveries than if the cells were treated with one or the other. Therefore, we took the results of the previous experiment to determine the appropriate CPA mixture solution as the best performing ratio. We determined that among the pCPAs, DMSO was the best performing due to a high initial post thaw recovery and greatest overall growth. Among the npCPAs, trehalose and PEG performed comparably when measuring initial post thaw recovery and proliferation, with cells treated with trehalose resulting in greater recovery; however, upon further testing, we found that cells treated with trehalose tended to be contaminated, possibly due to trehalose being a sugar that may easily allow for bacteria to grow. Therefore, the npCPA selected to proceed with the CPA mixture experiments was PEG.

hBEcs were treated with a mixture of DMSO and PEG in varying concentrations, both the overall concentration of CPA in each vial remained to be 10% v/v. Cells were harvested and aliquoted into cryovials, with respective amounts of DMSO + PEG shown in Table 3.

Table 3.

Combinations of DMSO and PEG in varying amounts.

DMSO (%)	PEG (%)	hBEC culture medium (%)
10	-	90
7.5	2.5	90
5	5	90
2.5	7.5	90
-	10	90

Cells were frozen for 3 days, and then initial post-thaw recovery was measured using a hemocytometer and trypan blue staining.

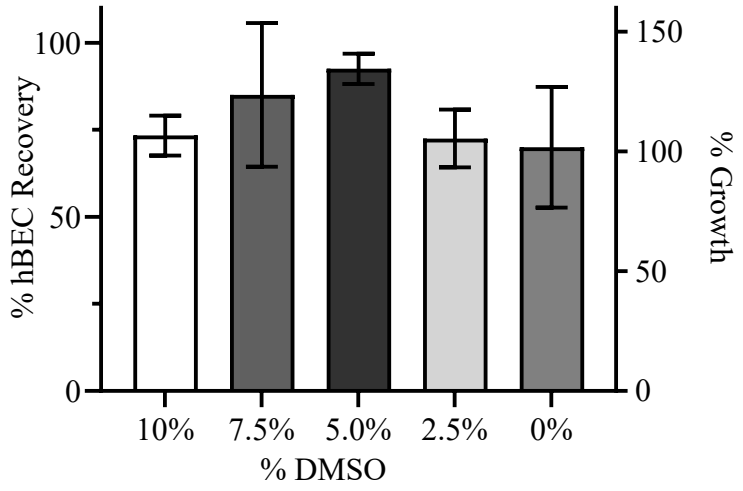

Figure 4. Initial recovery of hBECs following cryopreservation comprising of DMSO and PEG mixtures. The total amount of CPA remained to be 10% v/v; however, the amount of DMSO varied from 0%, 2.5%, 5.0%, 7.5%, and 10% DMSO, with PEG composing the remaining 10%, 7.5%, 5.0%, 2.5%, and 0%, respectively, for 7 days at -140°C. $p > 0.05$ compared to samples frozen with 10% DMSO.

While cells treated with the CPA mixture comprising of 5.0% DMSO and 5.0% PEG resulted in the highest post-thaw recovery, the increase was insignificant. The post thaw recoveries of hBECs treated with 10% DMSO and 0% DMSO (10% PEG) were consistent with that of Figure 1, and hBECs that were treated with 2.5% DMSO + 7.5% PEG as well as 7.5% DMSO + 2.5% PEG were only slightly higher. Based on the results, we determined that 10% DMSO or 10% PEG was still a suitable CPA solution for a recovery of 70%; any increases in recovery can be obtained due to mixing of the two CPAs.

4.4 Effect of ROCK Inhibitor on recovery

Previous research has shown that the incorporation of ROCK inhibitor in stem cell cryopreservation was shown to increase the recovery as well as proliferation. As part of a previous

protocol regarding the cryopreservation of stem cells, ROCK inhibitor was included into the freezing media prior to freezing, as well as into the culturing medium after thawing. Therefore, a 1000 μM solution of ROCK inhibitor in hBEC media was developed and filtered via vacuum filtration. The ROCK inhibitor solution was then further diluted into 100 μM, 50 μM, 10 μM, 5 μM, and 1 μM, and each dilution was added into the freezing medium containing 10% DMSO. Samples were frozen for 7 days at -140ºC and thawed at 37ºC; recovery was determined via hemocytometer and trypan blue staining.

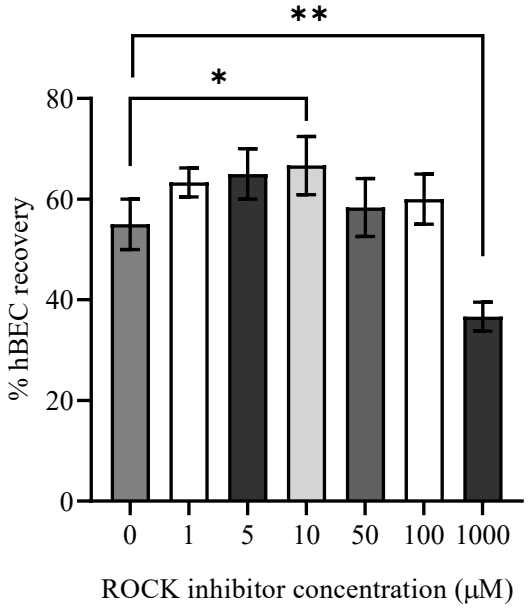

Figure 5. Initial post thaw recovery of cells treated with ROCK inhibitor and 10% DMSO. ROCK inhibitor was incorporated into hBEC suspensions at varying concentrations and samples were frozen for 7 days at -140ºC. Post thaw recovery was obtained via hemocytometer and trypan blue staining after thawing at 37ºC. *$p < 0.05$ and **$p < 0.01$ for samples treated with no ROCK inhibitor and 10% DMSO.

It was found that samples treated with 10 μM prior to freezing resulted in the greatest overall recovery. As shown in Figure 5, while there was an increase in recovery with the addition

of ROCK inhibitor, it appeared that was an optimal concentration that would result in the greatest recovery. Changes were insignificant when 50 μM and 100 μM was incorporated into the freezing mixture, and was found to actually significantly decrease recovery when adding 1000 μM.

4.5 Effect of IRIs on recovery

It has been previously researched that the addition of PVA has inhibitory effects on ice nucleation. As an ice recrystallization inhibitor, PVA can adsorb to the surface of ice crystals which can preemptively prevent any mechanical injury that results from additional ice crystal growth. A stock solution of 8 μM PVA in hBEC culture medium was developed and filtered by vacuum filtration. The stock solution was further diluted accordingly into 4 μM, 2 μM, 1 μM, and 0.5 μM, which was then added into the cell suspension mixture with CPA prior to freezing. The effects of PVA on hBECs' post-thawing recovery were measured after 7 days of cryopreservation.

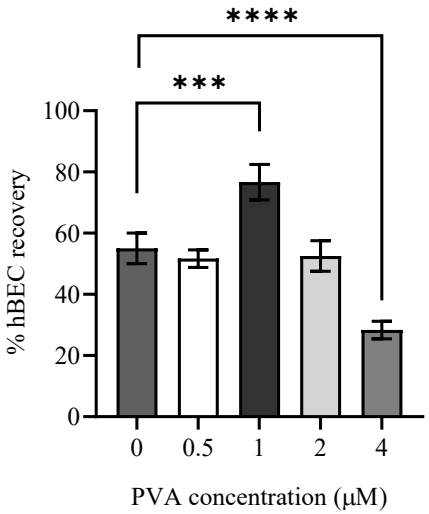

Figure 6. Initial recovery of hBECs treated with PVA in addition to 10% DMSO. Samples were evaluated for recovery via hemocytometer and trypan blue after being cryopreserved for 7 days and immediately thawing at 37°C. ***$p < 0.001$ and ****$p < 0.0001$ for samples frozen with 10% DMSO with no addition of PVA.

It was found that hBECs treated with 10% DMSO with 1 μM of PVA had a recovery that was significantly greater than the samples treated with PVA, including the control sample that was did not contain PVA in the freezing mixture. In addition, as shown in Figure 5, the addition of 1 μM to the freezing mixture was the optimal concentration, as additions of 0.5 μM and 2 μM PVA seemed to have an insignificant effect on the recovery, while greater concentrations of 4 μM actually resulted in a significant decrease in recovery.

Physical parameters of freezing

4.6 Effect of cooling rates on recovery

Previous research has shown that the rate of freezing has a pronounced effect on the recovery of cells. Typically, mammalian cells are frozen at a rate between -1° to -2°C, but it has been found that different cell lines tolerate have optimal cooling rates. Therefore, we investigated different cooling rates and temperatures while using DMSO as the primary CPA. While we did not have a programmable controlled-rate freezer, we determined that samples are commonly frozen at in a -80°C freezer, or stored in a liquid nitrogen container; within the liquid nitrogen container itself, samples stored towards the top are exposed to vaporous nitrogen at -140°C, while samples stored towards the bottom are exposed to liquid nitrogen at -196°C. In addition, common lab practices include moving the samples to colder temperatures for long-term storage. Therefore, we have tested 5 different freezing conditions.

Table 4.

hBEC freezing conditions and their respective durations.

Freezing Method
-196°C (liquid N_2, 7 days)
-140°C (vapor N_2, 7 days)
-80°C (7 days)
-140°C (overnight), -196°C (6 days)
-80°C (overnight), -140°C (overnight), -196°C (5 days)

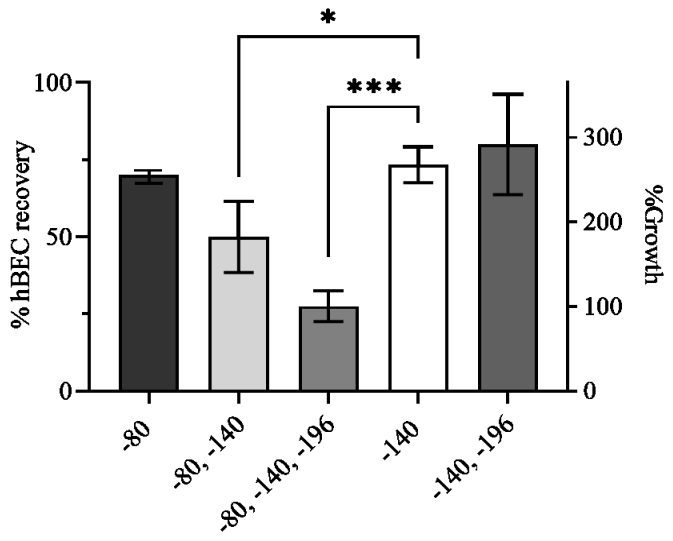

Figure 7. hBECs were aliquoted into cryovials at a density of 0.5 x 10^6 cells/mL containing 10% DMSO and moved into different freezing conditions. hBECs were either kept in their respective freezers or moved after 24 hours into a different temperature. After 7 days total of cryopreservation, post-thaw recovery was obtained via hemocytometer and trypan blue staining. *$p < 0.05$ and ***$p < 0.001$ for samples frozen at -140°C, the current standard.

As shown in Figure 7, hBECs frozen at -140°C were 150% times greater than those that were moved from -80°C to -140°C, and almost 300% times greater than those that were moved from -80°C to -140°C and finally -196°C. However, hBECs that remained at -80°C and those that

were moved from -140°C (vaporous N_2) to -196°C (liquid N_2) had recoveries comparable to the original freezing condition, which was to freeze the cells at -140°C; hBECs that were moved from vaporous N_2 to liquid N_2 did increase in recovery, but only slightly, and hBECs that remained at -80°C resulted in a slightly lower recovery.

4.7 Effect of cell density on recovery

In a protocol described by Freshney, hBECs were typically frozen at a cell density of 2.0 x 10^6 cells/mL[24]; however, with the inconsistent recovery and quantities of cells after cryopreservation, the possibility of losing a large number of cells becomes very likely. Therefore, the majority of our experiments have been performed using a cell density of 0.5 x 10^6 cells/mL. On the other hand, the cell density may have some impact in cell freezing, potentially due to the space in between cells that may affect ice formation. We varied the cell density as shown in Figure 8 and treated the samples with 10% DMSO, which were kept at -140°C for 7 days.

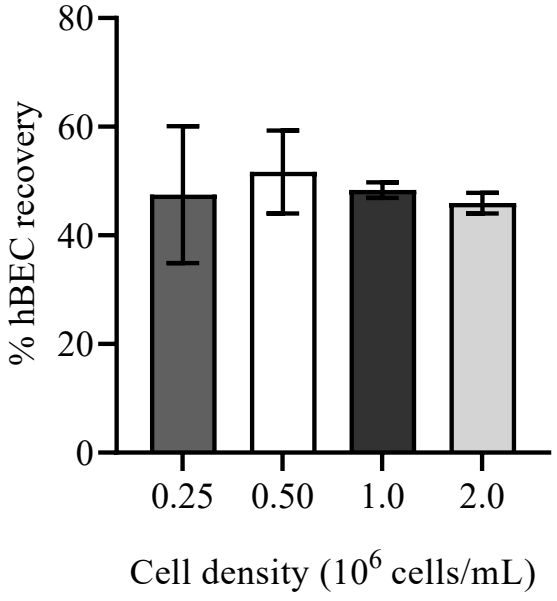

Figure 8. Initial post-thaw recovery of hBECs varying the cell density prior to freezing. hBECs were aliquoted into cryovials at different cell densities and treated with 10% DMSO. Samples were frozen at -140°C for 7 days, and post thaw recovery was obtained using hemocytometer and trypan blue staining. $p > 0.05$ compared to samples that are frozen at a cell density of 0.5×10^6 cells/mL.

As shown in Figure 8, cell density did not have any significant effect on the post thaw recovery. While there was a slight increase in recovery when freezing at a density of 0.5×10^6 cells/mL, there is no definitive advantage of freezing at one cell density over another. Therefore, the standard of freezing cells at a density of 0.5×10^6 cells/mL can still be implemented, but changes in cell density can ultimately be implemented with no detrimental effects to the recovery.

4.8 Effect of equilibration time on recovery

Immediately after the addition of CPAs, cells need to remain at room temperature for a certain amount of time to allow for the CPA to penetrate through the cell membrane, which we have deemed the "equilibration time". Therefore, the equilibration time is only applicable to pCPAs, which exhibit cryoprotective properties by permeating through the cell membrane to prevent intracellular ice formation and dehydration. We varied the amount of time that we exposed the hBECs to DMSO at room temperature prior to freezing. In prior experiments, we have been exposing the hBECs to equilibrate at room temperature for 15 minutes.

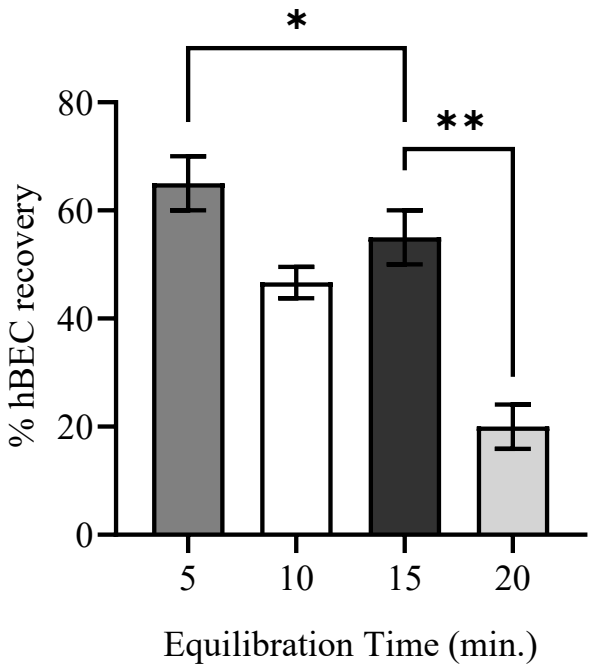

Figure 9. Initial post-thaw recovery of hBECs with varying equilibration times. hBEC suspensions were aliquoted into cryovials at a cell density of 0.5 x 10^6 cells/mL and treated with 10% DMSO. Immediately after addition of DMSO, samples remained at room temperature either 5, 10, 15, or 20 minutes, and were frozen at -140°C. Post thaw recovery was obtained by hemocytometer and trypan blue staining. *$p < 0.05$ and **$p < 0.01$ compared to samples that remained at room temperature for 15 minutes after CPA addition.

The equilibration time had a pronounced effect on the post thaw recovery of the hBECs. Samples that remained at room temperature for 5 minutes were significantly greater than those that remained at room temperature for 15 minutes. In addition, any increases in equilibration time resulted in significant decreases in recovery.

Chapter 5: Discussion

In tissue reconstruction of the bladder, hBECs are highly important in the development of the urothelium. Currently, hBECs are known to have a low recovery after cryopreservation and an

inconsistent growth, which proves to be experimentally problematic. While the cryopreservation of other epithelial cells has been optimized, effective cryopreservation of hBECs has not been thoroughly investigated as such. Ensuring for the greater recovery and shorter proliferation time to confluency allows for a more holistic understanding of the development of an engineered bladder, as well as providing insight to more effectively freeze bladder cells.

In our research, we froze hBECs while altering one of two factors: the chemical composition of the freezing solution and the physical parameters involved with freezing. For each experiment performed, we found the initial recovery of the cells immediately after thawing using a hemocytometer and trypan blue staining; for experiments that looked at proliferation, we seeded hBECs into 96 well plates in which we monitored their growth over an eight-day period. Initially, we tested seven different CPAs of both the penetrating and non-penetrating variety. From both post-thaw recovery and proliferation experiments, we concluded that DMSO and PEG resulted in the greatest recoveries of cells as well as the greatest growth of cells after an eight-day growth period. These results build on existing evidence of the protocol described by Freshney, such that treating hBECs with DMSO resulted in a recovery of >70% after cryopreservation[24]. However, our results also indicate that PEG is a suitable CPA alternative for hBECs that produces similar results without the known toxicity issues from DMSO. The use of PEG as a CPA may also have clinical implications when reconstructing the urothelium, such that trace amounts will have less severe of an effect compared to using DMSO as a CPA, which has been known to have issues with cell signaling[15].

It has also been previously reported that a mixture of pCPAs and npCPAs enhances recovery of cells following cryopreservation due to the decreased cell volume and increased osmolarity, and previously shown that a CPA mixture with DMSO as the penetrating compound

enhanced recovery compared to DMSO by itself[17,25,26]. These reports are only somewhat consistent with our results, as shown in Figure 4; while the mixture between 5.0% DMSO and 5.0% PEG resulted in the greatest recovery of 87%, the increases are insignificant and the recoveries are ultimately comparable to that of 10% DMSO or 10% PEG. Upon initial review, these minor increases in recovery allow for the more complete usage of the samples frozen, but the original recovery from hBECs treated with 10% DMSO or 10% PEG is still sufficient and may not warrant the extra costs, resources, and effort involved in using PEG as an additive to the freezing mixture. On the other hand, however, it is also confirmed that freezing mixtures comprised of both DMSO and PEG result in comparable, if not higher recovery. These results are still relevant to any sort of clinical applications, such that in the cryopreservation of these cells, one could potentially offset the toxicity that stems from DMSO by reducing its amount required with the addition of DMSO.

Previous studies have reported that dilute concentrations of ROCK inhibitor during culturing after cryopreservation has significantly increased the proliferation rate [18,27,28]. In addition, a previous protocol regarding the cryopreservation of stem cells implemented ROCK inhibitor in the freezing mixture as well as into the culturing medium. With the addition of ROCK inhibitor in the freezing mixture, our results showed that cells treated with 10 μM of ROCK inhibitor resulted in the greatest recovery. These results build on existing evidence that shows that there is an optimal concentration of ROCK inhibitor at which the cells have a greater recovery or growth[29]. While these experiments were performed with the intention of increasing recovery instead of increasing the proliferation rate, the methodological choices were constrained by the lack of consistent quantities and quality of hBECs when experimenting with ROCK inhibitor in the culturing medium. However, incorporation of ROCK inhibitor in the freezing medium did result in a

significant recovery, which may be investigated as a contributing factor in future experiments when investigating proliferation.

The results also indicate that the addition of PVA also had a profound effect on the recovery of the hBECs. As shown in Figure 6, cells treated with an optimal concentration of 1 μM PVA resulted in the greatest recovery of 76%, significantly higher than those that were untreated with PVA; in addition, too high of a concentration resulted in a significant decrease in the recovery following cryopreservation. These results are in line with the previous studies, which suggested that dilute amounts of PVA would allow for greater recovery[22,23].These results build on existing evidence that addition of PVA allows for the increase in recovery; the increases in recovery may be a result of a combination of the decreased toxic effects of DMSO, as well as the decreased mechanical injury that results from intracellular ice formation.

In terms of the physical parameters, we found that the equilibration time had a profound impact on the recovery of cells. Previously, after the addition of CPA, we let the cells remain at room temperature for 15 minutes; while this equilibration time would only apply to pCPAs, the results indicate that increases in equilibration time resulted in a decrease in recovery. While there has not been previous research done on the exact phenomenon, DMSO is known to be toxic to cells, and prolonged exposure to DMSO at room temperature would result in a decrease in recovery or viability. Therefore, an equilibration time of 5 minutes is a sufficient amount of time for DMSO to exhibit its cryoprotective effects as well as ensuring a higher recovery. In other previous research, the cooling rate and freezing temperature has also been a major factor in the survivability of cryopreserved cells. Previous research has found that there is an optimal rate at which cells are cryopreserved, which may vary among different cell lines[16,30,31]. While these reports may not immediately correlate with our findings of an optimal cooling rate or freezing temperature, our

data does imply that the cooling rate and duration spent at different temperatures has a pronounced effect on the post thaw recovery. hBECs that were moved from -80°C to N_2 storage resulted in the lowest recoveries among the freezing conditions, potentially due to a steep decrease in temperature rather than a gradual decrease that can be obtained through a controlled rate freezer. The low recovery in the hBECs that were moved from -80°C may have also resulted from any thawing that may have occurred in between transferring among different freezers; this result can also be supported by hBECs that were moved from vaporous N_2 to liquid N_2, as they may have had minimal exposure to external temperatures[30,31].

However, despite the consistency in higher recovery, our results cannot conclude whether the hBECs exhibited their specific markers and would have been able to function as a component of the urothelium. As mentioned previously, in many of our experiments following the initial screening of CPAs, we consistently found that hBECs that were seeded into 96 well plates for proliferation studies were inconsistent in their growth and degree of contamination, thereby giving us inconclusive data regarding the effect of CPA mixtures and ROCK inhibitor on the proliferation. In addition, the lack of a controlled rate freezer may have created some limitations in the study; a programmable cooling rate can be set to be constant for all conditions that were being tested, which could potentially exclude it as a factor that may have caused any discrepancies. A programmable cooling rate also allows for the possibility that an optimal cooling rate[34] that is the most suitable for hBECs.

Despite these limitations, each of the experiments provided insight as a method to potentially increase recovery immediately after thawing, as shown by the significant increases after treatment. Based on these data, further research is required to investigate long term viability of these cells, as well as the effects of cryopreservation on urothelial marker expression. Possible

future directions include the use of Raman spectroscopy to observe the cell morphology during freezing. In addition, other future directions include observing the sustainability and viability of hBECs in clinical applications, specifically to research the effects of different chemical compositions and freezing rates *in vivo*, and to eventually implement these cryopreserved cells in tissue regeneration.

We can therefore conclude that DMSO and PEG, perform comparably in terms of their cryoprotective capabilities, and treatment with mixtures of the two CPAs can also provide consistent recoveries. Addition of an optimal concentration of ROCK inhibitor or PVA prior to freezing also resulted in significantly higher recoveries. In terms of physical parameters, increases in time spent at room temperature following the addition of the freezing mixture will result in a decrease in recovery. Based on these data, we have developed a method that can successfully allow for a greater number of hBECs for use in further testing and potential future clinical applications.

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Curriculum Vitae

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PROFESSIONAL GOALS:

My overall goal is to pursue a career in cancer research, either in the field of treatment and diagnostics of cancer, or engineering the technology to help treat it. However, I am also generally interested in all aspects of medicine, research, and clinical applications that are currently being investigated as well as novel technologies being used to better understand mechanisms and better treatment outcome of human diseases.

EDUCATION:

Johns Hopkins University Baltimore, MD
Master of Science, Chemical and Biomolecular Engineering October 2019
Bachelor of Science, Chemical and Biomolecular Engineering May 2018

Relevant courses: Transport phenomena I + II, Pharmacokinetics, Pharmacodynamics, Chemical and Biomolecular Engineering Laboratory, Polymer Design and Bioconjugation, Applications of Molecular Evolution to Biotechnology, Statistical Thermodynamics, Biochemistry, Biochemistry Lab, Cell Biology, Process Design with ASPEN, Product Design

EXPERIENCE:

Brady Urological Institute – Johns Hopkins School of Medicine Baltimore, MD
Graduate Researcher 2018-Present

- Involved in the overall research to regenerate bladder tissue for the implementation in models that have carcinogenic, damaged, or removed bladders
- Researched ways to improve cryopreservation of human bladder epithelial cells, so that there are sufficient quantities for further experiments
- Assisted in animal surgeries involving bladder tissue regeneration, as well as subcutaneous tissue regeneration

American Gene Technologies Rockville, MD
Research Intern 2017-2018

- Involved in the overall research to develop new mechanisms to treat cancer with the use of retroviruses
- Researched the effects of gamma-delta T cells on different cancer cell lines, and analyzed these effects using various cytotoxicity assays and flow cytometry.
- Assisted in gene cloning through development of vectors, including PCR, dissection enzymes, and gel electrophoresis

Institute of NanoBioTechnology (Konstantopoulos Lab) – Johns Hopkins University Baltimore, MD
Undergraduate Researcher 2016-2018

- Researched the mechanisms of cell motility in two-dimensional unconfined spaces, and three-dimensional extracellular matrices
- Involved in the development of microfluidic devices that replicate the three-dimensional spaces
- Researched the effect of confined spaces on cytokinesis of various carcinogenic cell lines: HeLa, 3T3, HT1080

Department of Pediatrics (Atherosclerosis Unit) – Johns Hopkins School of Medicine Baltimore, MD
Undergraduate Researcher 2015-2016

- Researched the effects of the glycosphingolipid lactosylceramide (LacCer) on the body, using high performance liquid chromatography and mass spectrophotometry analysis
- Involved in the extraction of plasmids from bacteria to be transfected in cancerous cells
- Assisted in the maintenance of mice and harvesting of organs during the research process

ACTIVITIES AND LEADERSHIP:

Hopkins Emergence Response Organization Baltimore, MD
Crew Member 2015-2018

- Provided emergency medical care to the students and faculty at Johns Hopkins University Homewood campus
- Acts in coordination with Johns Hopkins security personnel to provide a safe environment

Alpha Phi Omega Baltimore, MD
Service Fraternity Member 2014-2018

- Offers to the greater community of Baltimore by providing care to homeless individuals, as well as contributing to the well-being and education for the Baltimore youth
- Assisted many outside groups in their efforts towards the betterment of the Baltimore community

United Innoworks – Johns Hopkins Chapter Baltimore, MD
Deputy Director 2014-2016

- Designed a summer enrichment program for Baltimore middle school students who come from low income families to help minimize achievement gap
- Worked in accordance with Baltimore public schools to provide outside tutoring and academic guidance to students

SKILLS:

Chinese, Mandarin: Intermediate written and conversational level
Microsoft Word/Powerpoint/Excel; Matlab; Java, AutoCAD
CPR/AED; Maryland EMT-B; NREMT